Materials and methods

In vitro differentiation

hPSCs harvested with GCDR were transferred to ultra-low attachment 6-well dish (Corning, NY 14831) to demonstrate three germ layer differentiation potential through the formation of embryoid bodies (EBs) for 6-14 days with hPSC medium, Dulbecco's modified Eagle's medium (D-MEM)/F12 (SIGMA, cat # D6421), supplemented with 20% knockout serum replacement (KSR; GIBCO, cat # 10828-028), 2 mM GlutaMax™ (Nacalai Tesque, 16948-04), 1% non-essential amino acids (GIBCO, cat # 11140-050), 0.1 mM 2-mercaptoethanol (GIBCO, cat # 21985), and penicillin-streptomycin (Nacalai Tesque, cat # 26253-84). EBs were transferred to 6-well dish coated with 0.1% gelatin (SIGMA, G1890) for RNA extraction or 12-well dish for IHC. Expression of lineage-specific genes was examined by IHC after 10-14 days. Molecules representing three germ layer differentiation were detected by IHC using antibodies against β-tubulin (ectoderm), glial fibrillary acidic protein (GFAP; ectoderm), vimentin (mesoderm), and desmin (mesoderm) and visualized with secondary antibodies labeled with Alexa Fluor 594 (Invitrogen™). Nuclei were stained with DAPI (Invitrogen™). A fluorescence microscope (OLYMPUS, IX71) was used for fluorescent observation. All antibodies for lineage-specific analysis are listed in Table S1.

Teratoma formation assay

hPSCs (4.0×10^6 cells) cultured over five passages after thawing were used for teratoma formation assays, performed by transplanting them under the epidermal space of the left testis of NSG (NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice (Charles river, Japan). Ten μL of HBSS buffer (Nacalai Tesque, 09735-75) was injected into the right testis as a negative control. All mice developed teratomas at the injection sites by 10-12 weeks. Teratomas that formed within testicles were fixed with 4% PFA and sectioned, and sections were stained with hematoxylin (Sakura Finetek, Japan) and eosin (Merck Millipore).

Table S1. List of antibodies used for FACS and IHC

Antibodies for Immunochemical staining		Supplier	Cat No.	dilution ratio
pluripotent marker	POU5F1	SantaCruze	sc-5279	1:200
	NANOG	Reprocell	RCAB0003P	1:200
	NANOG	Cell signaling	3580	1:800
	SSEA-4	Millipore	MAB4304	1:200
	TRA 1-60	Millipore	MAB4360	1:200
	TRA 1-81	Millipore	MAB4381	1:200
different marker	β-tubulin	SIGMA	T4026	1:200
	Glial fibrillary acidic protein (GFAP)	SantaCruze	sc-6170	1:100
	Desmin	Dako	M0760	1:100
	Vimentin	SIGMA	V6630	1:200
second antibody	Alexa Fluor 488 goat anti-rabbit IgG (H+L)	Invitrogen	A11008	1:500
	Alexa Fluor 594 rabbit anti-mouse IgG (H+L)	Invitrogen	A11062	1:500
	Alexa Fluor 594 donkey anti-goat IgG (H+L)	Invitrogen	A11058	1:500
	DAPI	Invitrogen	D1306	1:500
Antibodies for FACS		Supplier	Cat No.	
pluripotent marker	SSEA-3	BD	561145	
	SSEA-4	BD	560126	
	TRA 1-60	BD	562711	
	7AAD	BD	51-68981E	
Neu5Gc analysis	Anti-Neu5Gc antibody kit	BioLegend	146901	
	Alexa Fluor 647-AffiniPure F(ab')2 Fragment Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson ImmunoResearch	703-606-155	

Table S2. List of primers used for qRT-PCR

	Gene	Accession number	Sequence	
pluripotent marker	POU5F1	NM_001173531	F	GAAACCCACACTGCAGCAGA
			R	TCGCTTGCCCTTCTGGCG
	NANOG	NM_024865	F	CTCAGCTACAAACAGGTGAAGAC
			R	TCCCTGGTGGTAGGAAGAGTAAA
	SOX2	NM_003106	F	GGGAAATGGGAGGGGTGCAAAAGAGG
			R	TTGCGTGAGTGTGGATGGGATTGGTG
	KLF4	NM_004235	F	CGCTCCATTACCAAGAGCTCAT
			R	CGATCGTCTTCCCCTCTTTG
	MYC	NM_00246	F	CGTCTCCACACATCAGCACAA
			R	TCTTGGCAGCAGGATAGTCCTT
	TERT	NM_198253.2	F	CGTACAGGTTTCACGCATGTG
			R	ATGACGCGCAGGAAAAATGT
	NODAL	NM_00100	F	GAAATCCTCGCAACTCATCCA
			R	TGGAGTTGGTTACACGCACTGT
	LEFTY2	NM_00324	F	GCTCAGATGCTGAGCTCTAGTAGGA
			R	GAAACTCCCAGCTGAAAATGTGT
	REX1	NM_174900	F	TGCAGGCGGAAATAGAACCT
			R	TCATAGCACACATAGCCATCACAT
internal control	GAPDH	NM_001256799	F	CCACTCCTCCACCTTTGACG
			R	ATGAGGTCCACCACCCTGTT

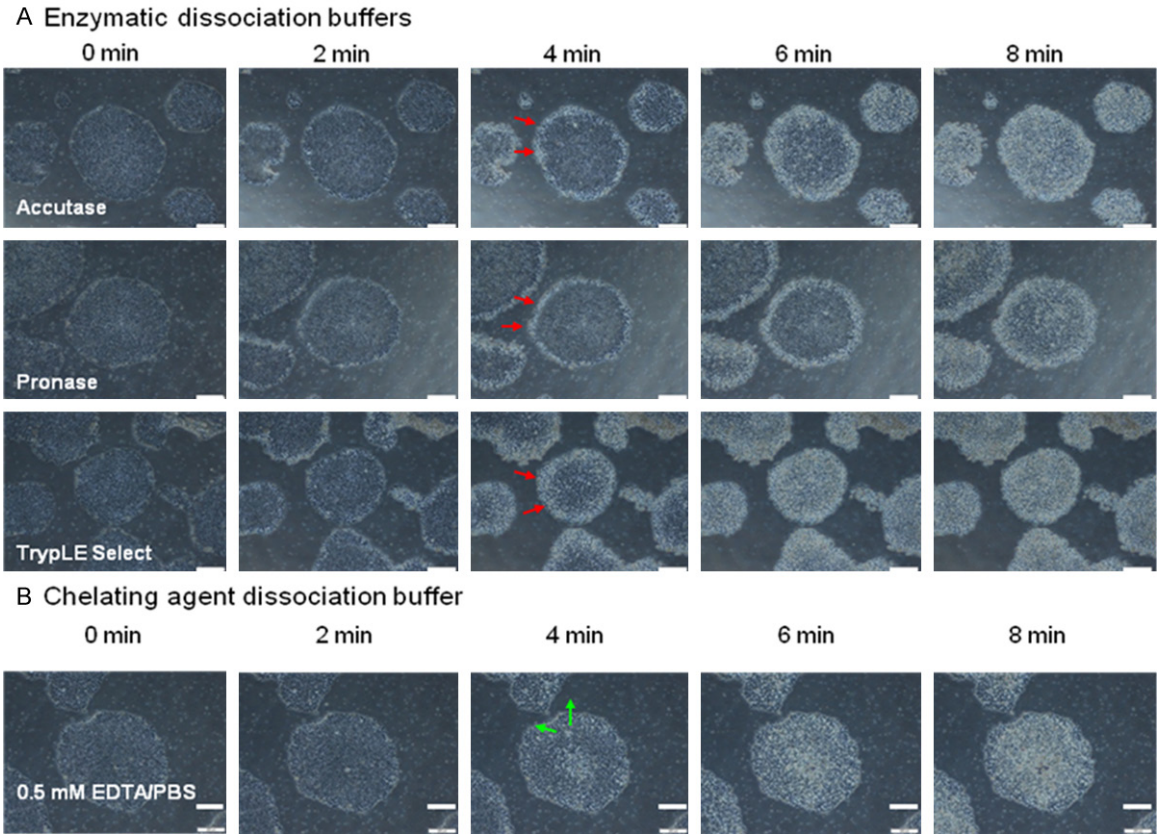

Figure S1. Time-lapse photographs of dissociation of KhES-1 cells on rhVTN-N. A. Time-lapse photographs of dissociation of KhES-1 cells on rhVTN-N treated with enzymatic dissociation reagents (Accutase™, Pronase, or TrypLE™ Select). Scale bar = 100 μm. Red arrows show that cell detachment initiated from the rims of the colonies. B. Time-lapse photographs of dissociation of KhES-1 cells on rhVTN-N treated with chelating agent EDTA/PBS(-). Green arrows show that cell detachment initiated from the centers of colonies.

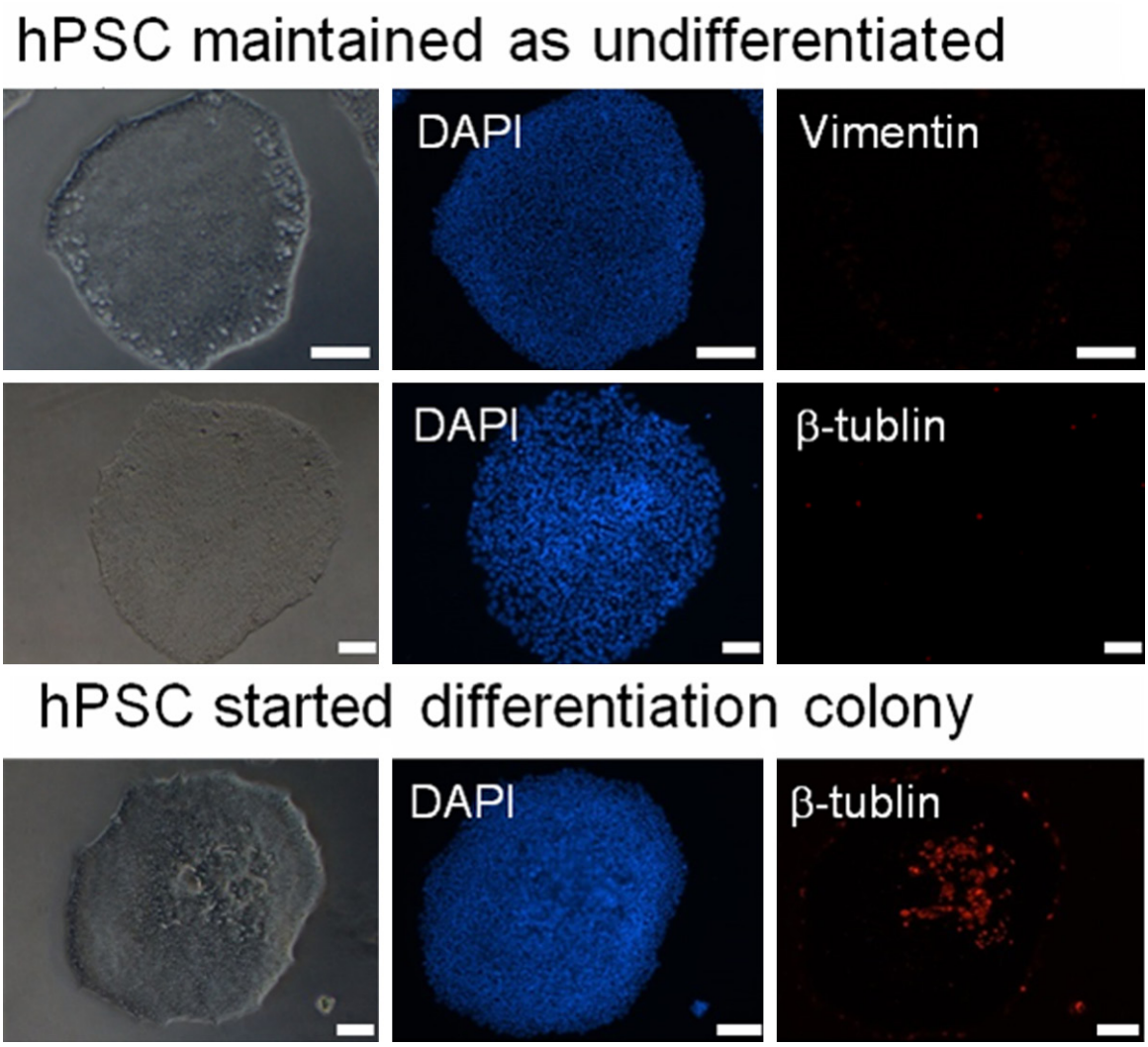

Figure S2. Detection of Vimentin and β-tublin in differentiated cells by ICH. When hPSC (KhES-1) were cultured as undifferentiated state. Mesodermal differentiation maker vimentin or ectodermal diffderentiation marker β-tublin was not detected by ICH (Upper panels). While, hPSC (KhES-1) once starts differentiated, Vimentin or β-tubilin was detected in differentiated cells in KhES-1 colony by IHC (lower panels). Bar = 100 μm

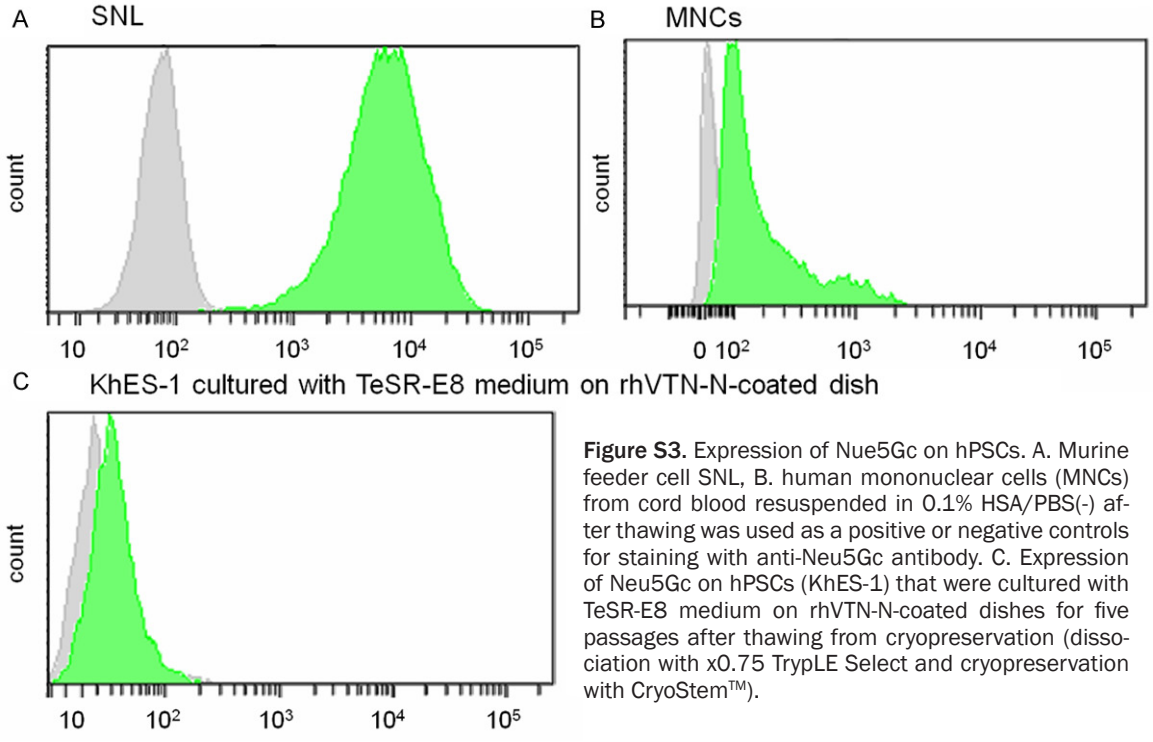

Figure S3. Expression of Nue5Gc on hPSCs. A. Murine feeder cell SNL, B. human mononuclear cells (MNCs) from cord blood resuspended in 0.1% HSA/PBS(-) after thawing was used as a positive or negative controls for staining with anti-Neu5Gc antibody. C. Expression of Neu5Gc on hPSCs (KhES-1) that were cultured with TeSR-E8 medium on rhVTN-N-coated dishes for five passages after thawing from cryopreservation (dissociation with x0.75 TrypLE Select and cryopreservation with CryoStem™).

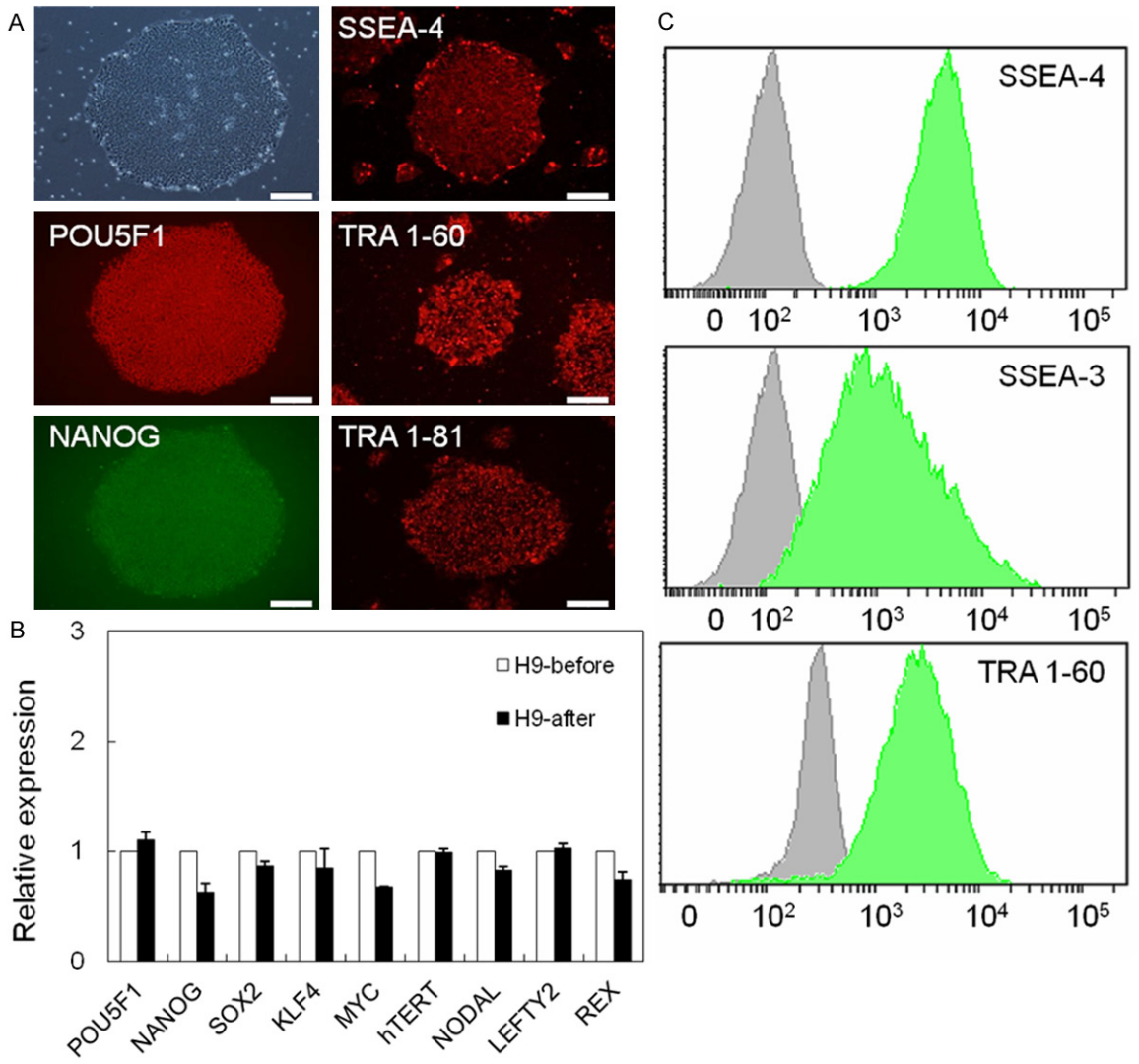

Figure S4. Self-renewal potential of thawed hPSCs. A. IHC of pluripotency-related molecules (POU5F1, SSEA-4, TRA 1-60, NANOG and TRA 1-81) in human ESCs (H9) at five passages after thawing. These molecules were detected byrespective antibodi and visualized with secondary Alexa Fluor 488 (green)- or 594 (red)-labeled antibodies. Scale bars = 200 μm. Phase contrast image of H9 is shown in upper left. B. Pluripotency-related genes (*POU5F1, NANOG, SOX2, KLF4, cMYC, hTERT, NODAL, LEFTY, and REX1*) in ES cell line H9 before (H9-before) and six passages after thawing (H9-after) were examined by qRT-PCR. Gene expression was compared by the Ct method. C. Flow cytometric analysis of pluripotency-related surface markers (SSEA-3, SSEA-4, and TRA 1-60) in iPS cell line H9 at four passages after thawing.

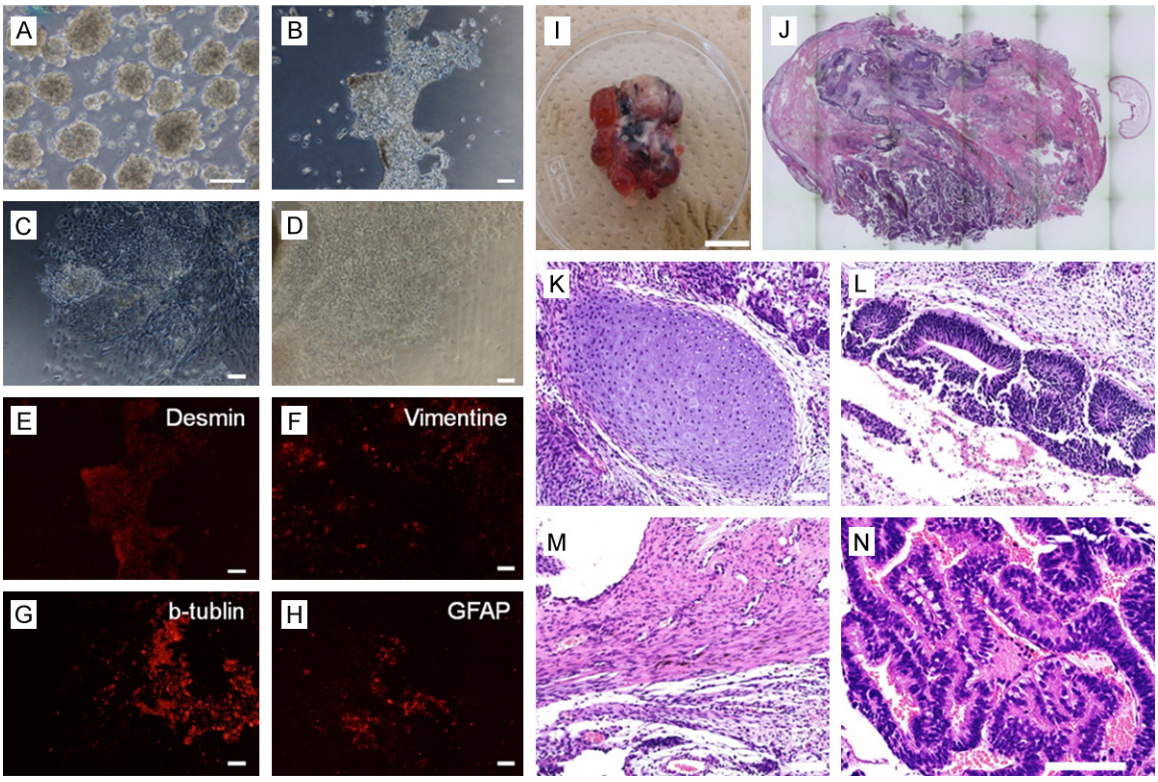

Figure S5. Differentiation potential of thawed hPSCs. A. Embryoid body (EBs) formation by KhES-1 cells six passages after thawing. B-D. Adherent cells with various morphologies on 0.1% gelatin-coated dishes from EBs. IHC with E. Desmin (mesoderm), F. Vimentin (mesoderm), G. β-tubulin (ectoderm) or H. GFAP (ectoderm). Alexa Fluor 546 (red) was used to label secondary antibodies. A-H. Scale bars = 100 μm. Teratomas from KhES-1 cells six passages after thawing in NSG mice. I. Whole teratoma. Scale bar = 10 mm, J. HE staining of teratoma in cross section, K. Cartilage, L. Neural tube, M. Muscle-like, N. Gut-like tissue in teratoma. K-N. Scale bar = 100 μm.